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(54) Title: USE OF CONOTOXIN PEPTIDES U002 AND MII FOR TREATING OR DETECTING SMALL-CELL LUNG CARCINOMA

(57) Abstract

The present invention is directed to use of relatively short peptides, spefically the α-conotoxin peptides MII and U002, for treating patients with small-cell lung carcinoma (SCLC) or for detecting the presence of SCLC tumors. It has been discovered that while MII and U002 bind to neuronal nicotinic receptors as do other α-conotoxin peptides, they have a significantly lower affinity for neuromuscular receptors. Patients having SCLC are treated in accordance with the present invention by administering, preferably intravenously or intramuscularly, a pharmaceutical composition containing the α-conotoxin peptide as the active ingredient. The presence or location of SCLC tumors are detected in accordance with the present invention by injecting a subject with MII or U002 labeled with a marker capable of detection and subsequently detecting the binding of the labeled MII or U002 to determine the presence or location of SCLC tumors.

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TITLE OF THE INVENTION

USE OF CONOTOXIN PEPTIDES U002 AND MII FOR TREATING OR DETECTING SMALL-CELL LUNG CARCINOMA

This invention was made with Government support under Grant No. GM-22737 awarded by the National Institutes of Health, Bethesda, Maryland. The United States Government has certain rights in the invention.

BACKGROUND OF THE INVENTION

This invention relates to the use of relatively short peptides, particularly the MII and U002 α-conotoxins, for treating patients with small-cell lung carcinoma (SCLC) or for detecting the presence of SCLC tumors.

The publications and other materials used herein to illuminate the background of the invention, and in particular, cases to provide additional details respecting the practice, are incorporated by reference, and for convenience are numerically referenced in the following text and respectively grouped in the appended bibliography.

Mollusks of the genus Conus produce a highly toxic venom which enables them to carry out their unique predatory lifestyle. Prey are immobilized by the venom which is injected by means of a highly specialized venom apparatus, a disposable hollow tooth which functions both in the manner of a harpoon and a hypodermic needle.

Few interactions between organisms are more striking than those between a venomous animal and its envenomated victim. Venom may be used as a primary weapon to capture prey or as a defense mechanism. These venoms disrupt essential organ systems in the envenomated animal, and many of these venoms contain molecules directed to receptors and ion channels of neuromuscular systems.

Predatory cone snails (Conus) have developed a unique biological strategy. Their venom contains relatively small peptides that are targeted to various neuromuscular receptors and may be equivalent in their pharmacological diversity to the alkaloids of plants or secondary metabolites of microorganisms. Many of these peptides are among the smallest nucleic acid-encoded translation products having defined conformations, and as such, they are somewhat unusual. Peptides in this size range normally equilibrate among many conformations. Proteins having a fixed conformation are generally much larger.

Cone snails that produce these toxic peptides, which are generally referred to as conotoxins or conotoxin peptides, are a large genus of venomous gastropods comprising approximately 500 species. All cone snail species are predators that inject venom to capture prey, and the spectrum of animals that the genus as a whole can envenomate is broad. A wide variety of hunting strategies are used; however, every Conus species uses fundamentally the same basic pattern of envenomation.

The major paralytic peptides in these fish-hunting cone venoms were the first to be identified and characterized. In C. geographus venom, three classes of disulfide-rich peptides were found: the α-conotoxin peptides (which target and block the nicotinic acetylcholine receptors); the μ-conotoxin peptides (which target and block the skeletal muscle Na^+ channels); and the ω-conotoxin peptides (which target and block the presynaptic neuronal Ca^{2+} channels). However, there are multiple homologs in each toxin class; for example, there are at least five different w-conotoxin peptides present in C. geographus venom alone. Considerable variation in sequence is evident, and when different ω-conotoxin peptide sequences were first compared, only the cysteine residues that are involved in disulfide bonding and one glycine residue were found to be invariant. Another class of conotoxins found in C. geographus venom is that referred to as conantokins, which cause sleep in young mice and hyperactivity in older mice and are targeted to the NMDA receptor. Each cone venom appears to have its own distinctive group, or signature, of different conotoxin sequences.

Many of these peptides have now become fairly standard research tools in neuroscience. μ-Conotoxin peptides, because of their ability to preferentially block muscle but not axonal Na^+ channels, are convenient tools for immobilizing skeletal muscle without affecting axonal or synaptic events. ω-Conotoxin peptides have become standard pharmacological reagents for investigating voltage-sensitive Ca^{2+} channels and are used to block presynaptic termini and neurotransmitter release. The α-conotoxins have various clinical uses. One such use is their utility as clinical muscle relaxants because of their ability to achieve antagonistic blockage of the mammalian neuromuscular junction nAChRs.

Small cell lung carcinoma (SCLC) cells have been found to express cholinergic nicotinic receptors (Maneckjee et al. (19); Chini et al. (20); Tarroni et al. (21); Schuller et al. (22)). These SCLC nicotinic receptors have been shown to be of neuronal type (Chini et al. (20); Tarroni et al. (21)). Nicotine and cytosine each stimulate the release of 5-hydroxytryptamine (5HT or serotonin)

which acts as a potent mitogen in SCLC cells (Maneckjee et al. (19); Cattaneo et al. (23)). α-Conotoxin MI has been found to block the nicotine or cytosine induced release of serotonin and at a concentration of 1 μM it completely antagonized the nicotine and cytosine stimulation of SCLC proliferation (Codignola et al. (24)). α-Conotoxins which bind to neuronal type nicotinic receptors are suitable for preventing the proliferation of tumors such as SCLC and can be used therapeutically to inhibit such proliferation as described below. These α-conotoxins can also be used diagnostically for detecting the presence and/or location of small-cell lung tumors as described below. Although Codignola et al. (24) report that α-conotoxin MI binds to these SCLC receptors, α-conotoxin MI is not suitable for therapeutic or diagnostic use since it also binds to neuromuscular receptors and can cause paralysis which could lead to death. α-Conotoxins which do not bind to neuromuscular receptors or which have a much lower affinity for such receptors as compared to the nicotinic neuronal receptors are suitable for therapeutic or diagnostic purposes.

Additional conotoxin peptides having the different general properties described above and conotoxin peptides which have lower affinity for neuromuscular receptors for treating small-cell lung carcinoma, continue to be sought.

SUMMARY OF THE INVENTION

The present invention is directed to use of relatively short peptides, specifically the α-conotoxin peptides MII and U002, for treating patients with small-cell lung carcinoma (SCLC) or for detecting the presence of SCLC tumors. It has been discovered that while MII and U002 bind to neuronal nicotinic receptors as do other α-conotoxin peptides, they have a significantly lower affinity for neuromuscular receptors.

Patients having SCLC are treated in accordance with the present invention by administering a pharmaceutical composition containing the α-conotoxin peptide as the active ingredient. The composition will generally contain a dosage unit, e.g., tablet, capsule, powder, injection, teaspoonful and the like, from about 200 to about 2000 nanomoles, and preferably 500 nanomoles of the active ingredient. The preferred mode of administration is intravenous or intramuscular. The dosing schedule may range anywhere from twice per day to once every few days, depending on the biological lifetime of the specific α-conotoxin peptide utilized.

The presence or location of SCLC tumors are detected in accordance with the present invention by injecting a subject with an MII or U002 labeled with a marker capable of detection and subsequently detecting the binding of the labeled MII or U002 to determine the presence or location of SCLC tumors.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Fish-hunting cone snails use a variety of paralytic peptides for envenomating their prey. The first Conus peptide isolated and characterized was a 13-amino-acid basic peptide from the venom of Conus geographus, α-conotoxin GI, which inhibits the nicotinic acetylcholine receptor at the neuromuscular junction of vertebrates. α-Conotoxins are used by a number of fish-hunting Conus species to block the neuromuscular junction of their prey. Six α-conotoxins from three different fish-hunting Conus species have been biochemically characterized.

All α-conotoxins purified from Conus venoms to date (shown in Table I) have several common structural features; there are 12 "core" amino acids that define the minimal functional unit for a high affinity α-conotoxin; the consensus sequence from the six different α-conotoxins is indicated in the Table. The most unusual α-conotoxin is α-conotoxin SII from Conus striatus, in which an additional disulfide bond is present. However, within the core sequence of all α-conotoxins, two disulfide bonds and a number of other amino acids are highly conserved.

TABLE I
α-Conotoxin Peptides

Peptide	Sequence	SEQ ID NO:
GI	ECCNPACGRHYSC*	1
GIA	ECCNPACGRHYSCGK	2
GII	ECCHPACGKHFSC*	3
MI	GRCCHPACGKNYSC*	4
SI	ICCNPACGPKYSC*	5
SIA	YCCHPACGKNFDC*	6
SII	GCCCNPACGPNYGCGTSCS	7
Consensus Core Sequence	N F CCHPACGXXYXC	8

* C-terminus is amidated.

Recently, predicted precursor structures for α-conotoxins from C. geographus were determined by a sequence analysis of cDNA clones encoding α-conotoxin GI and its homologs. The precursor of GI is a prepropeptide of 64 amino acids. For Conus peptides in general, the signal sequence and the 3' untranslated region adjacent the open reading frame are highly conserved. PCR primers were made using these α-conotoxin GI sequences, with the aim of deducing the sequences of additional α-conotoxin homologs from other Conus venoms. The strategy was to start with either messenger RNA or a cDNA library from a particular Conus venom duct, and to selectively amplify sequences related to the α-conotoxins.

The present invention is directed to use of relatively short peptides, specifically the α-conotoxin peptides MII and U002, for treating patients with small-cell lung carcinoma (SCLC) or for detecting the presence of SCLC tumors. It has been discovered that while MII and U002 bind to neuronal nicotinic receptors as do other α-conotoxin peptides, they have a significantly lower affinity for neuromuscular receptors. Thus, MII and U002 are particularly suited for the diagnosis and treatment of SCLC.

Patients having SCLC are treated in accordance with the present invention by administering a pharmaceutical composition containing the α-conotoxin peptide as the active ingredient. The composition will generally contain a dosage unit, e.g., tablet, capsule, powder, injection, teaspoonful and the like, from about 200 to about 2000 nanomoles, and preferably 500 nanomoles of the active ingredient. The preferred mode of administration is intravenous or intramuscular. The dosing schedule may range anywhere from twice per day to once every few days, depending on the biological lifetime of the specific α-conotoxin peptide utilized.

The presence of location of SCLC tumors are detected in accordance with the present invention by injecting a subject with an MII or U002 labeled with a marker capable of detection and subsequently detecting the binding of the labeled MII or U002 to determine the presence or location of SCLC tumors.

The A-lineage conotoxin peptides are conotoxin peptides that have strong homology in the signal sequence and the 3'-untranslated region of the genes coding for these peptides to the sequences in the α-conotoxin peptides. The A-lineage conotoxin peptides can be identified by performing polymerase chain reaction (PCR) amplification of Conus cDNA libraries or cDNA prepared by reverse transcription of venom duct mRNA, using primers based on the signal

sequence and the 3' untranslated region. The A-lineage conotoxin peptides include the α-conotoxin peptides, the α-conotoxin-like peptides and the κ-conotoxin peptides.

Specifically, the α-conotoxin peptides useful in the present invention have the following sequence:

U002: Gly-Cys-Cys-Ser-Asp-Pro-Arg-Cys-Ala-Trp-Arg-Cys (SEQ ID NO:9). The C-terminus is preferably amidated.

MII: Gly-Cys-Cys-Ser-Asn-Xaa$_1$-Val-Cys-His-Leu-Glu-His-Ser-Asn-Leu-Cys (SEQ ID NO:10). Xaa$_1$ may be Pro or hydroxy-Pro, and the C-terminus is preferably amidated.

Conotoxin peptides purified from Conus species generally contain hydroxy-Pro in place of Pro at many of the prolyl residues. Conotoxin peptides synthesized with either Pro or hydroxy-Pro have the biological activities described herein. Thus, Pro or hydroxy-Pro may be used at any prolyl or hydroxy-prolyl residues of the peptides identified herein, and they are considered to be equivalents. In addition to the post-translational processing of conotoxin peptides to modify prolyl residues to hydroxy-Pro, other residues are also post-translationally modified in the snail. These residues include Glx or Asx, which may be modified to γ-carboxyglutamate or β-carboxyaspartate, respectively. Such modification is seen when the residue is at the N-terminus of the conotoxin peptide. Additional post-translational modification may involve the glycosylation of Ser and/or Thr residues. Accordingly, conotoxin peptides having these modifications are considered to be equivalents of the sequences specified above and within the scope of the present invention.

After identification of the amino acid sequence of the conotoxin peptide, such as by purification and sequence analysis, PCR amplification, recombinant DNA techniques or the like, the mature conotoxin peptide can be synthesized using conventional techniques as described further below. DNA coding for U002 and MII (previously also termed MG-1) can be obtained as described in WO 95/11256 (18).

These peptides are sufficiently small to be chemically synthesized. General chemical syntheses for preparing the foregoing peptides are described hereinafter. An example of the specific chemical synthesis of one α-conotoxin peptide is shown in WO 95/11256. These conotoxin peptides can also be obtained by isolation and purification from specific Conus species using the techniques described in U.S. Patent No. 4,447,356 (1) or Olivera et al. (2).

Although the conotoxin peptides can be obtained by purification from the enumerated cone snails, because the amounts of conotoxin peptides obtainable from individual snails are very small,

the desired substantially pure conotoxin peptides are best practically obtained in commercially valuable amounts by chemical synthesis. For example, the yield from a single cone snail may be about 10 micrograms or less of A-lineage conotoxin peptide. By "substantially pure" is meant that the peptide is present in the substantial absence of other biological molecules of the same type; it is preferably present in an amount of at least about 85% by weight and preferably at least about 95% of such biological molecules of the same type which are present (i.e., water, buffers and innocuous small molecules may be present). Chemical synthesis of biologically active conotoxin peptides depends of course upon correct determination of the amino acid sequence.

The conotoxin peptides can also be produced by recombinant DNA techniques well known in the art. Such techniques are described by Sambrook et al. (3) The peptides produced in this manner are isolated, reduced if necessary, and oxidized to form the correct disulfide bonds.

One method of forming disulfide bonds in the A-lineage conotoxin peptides of the present invention is the air oxidation of the linear peptides for prolonged periods under cold room temperatures. This procedure results in the creation of a substantial amount of the bioactive, disulfide-linked peptides. The oxidized peptides are fractionated using reverse-phase high performance liquid chromatography (HPLC) or the like, to separate peptides having different linked configurations. Thereafter, either by comparing these fractions with the elution of the native material or by using a simple assay, the particular fraction having the correct linkage for maximum biological potency is easily determined. It is also found that the linear peptide, or the oxidized product having more than one fraction, can sometimes be used for in vivo administration because the cross-linking and/or rearrangement which occurs in vivo has been found to create the biologically potent conotoxin molecule. However, because of the dilution resulting from the presence of other fractions of less biopotency, a somewhat higher dosage may be required.

A second method of forming the disulfide bonds in the A-lineage conotoxin peptides of the present invention involves the use of acetamidomethyl (Acm) as protection agent on the second and fifth cysteines during the synthesis of the A-lineage conotoxin peptides. Use of Acm on these two residues is based on the analogy with disulfide bridges in other A-lineage conotoxin peptides. The peptide with the Acm protected cysteines is air-oxidized overnight at room temperature. The bicyclic peptides are separated by HPLC and the desired isomer isolated. The final disulfide bridge is carried out by iodination. The undesired isomers are efficiently recycled by reduction to linear peptide. The desired isomer is determined by a partial reduction analysis (4). In this analysis, a

sample of a bicyclic precursor is treated with tris-[2-carboxyethyl]phosphine to give linear peptide and a singly-bridged intermediate. The latter peptide is reacted with iodoacetamide, and the location of alkylated cysteine residues is established by sequence analysis.

The peptides are synthesized by a suitable method, such as by exclusively solid-phase techniques, by partial solid-phase techniques, by fragment condensation or by classical solution couplings. The employment of recently developed recombinant DNA techniques may be used to prepare these peptides, particularly the longer ones containing only natural amino acid residues which do not require post-translational processing steps.

In conventional solution phase peptide synthesis, the peptide chain can be prepared by a series of coupling reactions in which the constituent amino acids are added to the growing peptide chain in the desired sequence. The use of various N-protecting groups, e.g., dicyclohexylcarbodiimide or carbonyldimidazole, various active esters, e.g., esters of N-hydroxyphthalimide or N-hydroxy-succinimide, and the various cleavage reagents, to carry out reaction in solution, with subsequent isolation and purification of intermediates, is well known classical peptide methodology. Classical solution synthesis is described in detail in the treatise, "Methoden der Organischen Chemie (Houben-Weyl): Synthese von Peptiden," (5). Techniques of exclusively solid-phase synthesis are set forth in the textbook, "Solid-Phase Peptide Synthesis," (6), and are exemplified by the disclosure of U.S. Patent No. 4,105,603 (7). The fragment condensation method of synthesis is exemplified in U.S. Patent No. 3,972,859 (8). Other available syntheses are exemplified by U.S. Patents No. 3,842,067 (9) and 3,862,925 (10).

Common to such chemical syntheses is the protection of the labile side chain groups of the various amino acid moieties with suitable protecting groups which will prevent a chemical reaction from occurring at that site until the group is ultimately removed. Usually also common is the protection of an α-amino group on an amino acid or a fragment while that entity reacts at the carboxyl group, followed by the selective removal of the α-amino protecting group to allow subsequent reaction to take place at that location. Accordingly, it is common that, as a step in such a synthesis, an intermediate compound is produced which includes each of the amino acid residues located in its desired sequence in the peptide chain with appropriate side-chain protecting groups linked to various ones of the residues having labile side chains.

As far as the selection of a side chain amino protecting group is concerned, generally one is chosen which is not removed during deprotection of the α-amino groups during the synthesis.

However, for some amino acids, e.g., His, protection is not generally necessary. In selecting a particular side chain protecting group to be used in the synthesis of the peptides, the following general rules are followed: (a) the protecting group preferably retains its protecting properties and is not split off under coupling conditions, (b) the protecting group should be stable under the reaction conditions selected for removing the α-amino protecting group at each step of the synthesis, and (c) the side chain protecting group must be removable, upon the completion of the synthesis containing the desired amino acid sequence, under reaction conditions that will not undesirably alter the peptide chain.

It should be possible to prepare many, or even all, of these peptides using recombinant DNA technology. However, when peptides are not so prepared, they are preferably prepared using the Merrifield solid-phase synthesis, although other equivalent chemical syntheses known in the art can also be used as previously mentioned. Solid-phase synthesis is commenced from the C-terminus of the peptide by coupling a protected α-amino acid to a suitable resin. Such a starting material can be prepared by attaching an α-amino-protected amino acid by an ester linkage to a chloromethylated resin or a hydroxymethyl resin, or by an amide bond to a benzhydrylamine (BHA) resin or paramethylbenzhydrylamine (MBHA) resin. Preparation of the hydroxymethyl resin is described by Bodansky et al. (11). Chloro-methylated resins are commercially available from Bio Rad Laboratories (Richmond, CA) and from Lab. Systems, Inc. The preparation of such a resin is described by Stewart et al. (6). BHA and MBHA resin supports are commercially available, and are generally used when the desired polypeptide being synthesized has an unsubstituted amide at the C-terminus. Thus, solid resin supports may be any of those known in the art, such as one having the formulae -O-CH_2-resin support, -NH BHA resin support, or -NH-MBHA resin support. When the unsubstituted amide is desired, use of a BHA or MBHA resin is preferred, because cleavage directly gives the amide. In case the N-methyl amide is desired, it can be generated from an N-methyl BHA resin. Should other substituted amides be desired, the teaching of U.S. Patent No. 4,569,967 (12) can be used, or should still other groups than the free acid be desired at the C-terminus, it may be preferable to synthesize the peptide using classical methods as set forth in the Houben-Weyl text (5).

The C-terminal amino acid, protected by Boc and by a side-chain protecting group, if appropriate, can be first coupled to a chloromethylated resin according to the procedure set forth in K. Horiki et al. (13), using KF in DMF at about 60°C. for 24 hours with stirring, when a peptide

having free acid at the C-terminus is to be synthesized. Following the coupling of the BOC-protected amino acid to the resin support, the a-amino protecting group is removed, as by using trifluoroacetic acid (TFA) in methylene chloride or TFA alone. The deprotection is carried out at a temperature between about 0°C. and room temperature. Other standard cleaving reagents, such as HCl in dioxane, and conditions for removal of specific α-amino protecting groups may be used as described in Schroder & Lubke (14).

After removal of the α-amino-protecting group, the remaining α-amino- and side chain-protected amino acids are coupled step-wise in the desired order to obtain the intermediate compound defined hereinbefore, or as an alternative to adding each amino acid separately in the synthesis, some of them may be coupled to one another prior to addition to the solid phase reactor. Selection of an appropriate coupling reagent is within the skill of the art. Particularly suitable as a coupling reagent is N,N'-dicyclohexylcarbodiimide (DCC).

The activating reagents used in the solid phase synthesis of the peptides are well known in the peptide art. Examples of suitable activating reagents are carbodiimides, such as N,N'-diisopropylcarbodiimide and N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide. Other activating reagents and their use in peptide coupling are described by Schroder & Lubke (14) and Kapoor (15).

Each protected amino acid or amino acid sequence is introduced into the solid-phase reactor in about a twofold or more excess, and the coupling may be carried out in a medium of dimethylformamide (DMF): CH_2Cl_2 (1:1) or in DMF or CH_2Cl_2 alone. In cases where intermediate coupling occurs, the coupling procedure is repeated before removal of the α-amino protecting group prior to the coupling of the next amino acid. The success of the coupling reaction at each stage of the synthesis, if performed manually, is preferably monitored by the ninhydrin reaction, as described by Kaiser et al. (16). Coupling reactions can be performed automatically, as on a Beckman 990 automatic synthesizer, using a program such as that reported in Rivier et al. (17).

After the desired amino acid sequence has been completed, the intermediate peptide can be removed from the resin support by treatment with a reagent, such as liquid hydrogen fluoride, which not only cleaves the peptide from the resin but also cleaves all remaining side chain protecting groups and also the α-amino protecting group at the N-terminus if it was not previously removed to obtain the peptide in the form of the free acid. If Met is present in the sequence, the

Boc protecting group is preferably first removed using trifluoroacetic acid (TFA)/ethanedithiol prior to cleaving the peptide from the resin with HF to eliminate potential S-alkylation. When using hydrogen fluoride for cleaving, one or more scavengers such as anisole, cresol, dimethyl sulfide and methylethyl sulfide are included in the reaction vessel.

Cyclization of the linear peptide is preferably affected, as opposed to cyclizing the peptide while a part of the peptidoresin, to create bonds between Cys residues. To effect such a disulfide cyclizing linkage, fully protected peptide can be cleaved from a hydroxymethylated resin or a chloromethylated resin support by ammonolysis, as is well known in the art, to yield the fully protected amide intermediate, which is thereafter suitably cyclized and deprotected. Alternatively, deprotection, as well as cleavage of the peptide from the above resins or a benzhydrylamine (BHA) resin or a methylbenzhydrylamine (MBHA), can take place at 0°C. with hydrofluoric acid (HF), followed by oxidation as described above.

Patients having SCLC are treated in accordance with the present invention by administering a pharmaceutical composition containing the α-conotoxin peptide as the active ingredient. The composition will generally contain a dosage unit, e.g., tablet, capsule, powder, injection, teaspoonful and the like, from about 200 to about 2000 nanomoles, and preferably 500 nanomoles of the active ingredient. The preferred mode of administration is intravenous or intramuscular. In general α-conotoxin peptides are relatively resistant to degradation and may last on the order of several days in the body. Therefore, he dosing schedule may range anywhere from twice per day to once every few days, depending on the biological lifetime of the specific α-conotoxin peptide utilized.

Pharmaceutical compositions containing a compound of the present invention as the active ingredient in intimate admixture with a pharmaceutical carrier can be prepared according to conventional pharmaceutical compounding techniques. The carrier may take a wide variety of forms depending on the form of preparation desired for administration, e.g., intravenous, oral or parenteral In preparing the compositions in oral dosage form, any of the usual pharmaceutical media may be employed, such as, for example, water, glycols, oils, alcohols, flavoring agents, preservatives, coloring agents and the like in the case of oral liquid preparations (such as, for example, suspensions, elixirs and solutions); or carriers such as starches, sugars, diluents, granulating agents, lubricants, binders, disintegrating agents and the like in the case of oral solid preparations (such as, for example, powders, capsules and tablets). Because of their ease in

administration, tablets and capsules represent the most advantageous oral dosage unit form, in which case solid pharmaceutical carriers are obviously employed. If desired, tablets may be sugar-coated or enteric -coated by standard techniques. for parenterals, the carrier will usually comprise sterile water, though other ingredients, for example, to aid solubility or for preservative purposes, may be included. Injectable suspensions may also be prepared, in which case appropriate liquid carriers, suspending agents and the like may be employed.

The U002 or MII conotoxin is labeled with one or more radioisotope by standard techniques well known in the art. Any suitable radioisotope which can be scanned *in vivo* may be utilized. It is preferred to use ^{131}I or ^{125}I as the label. The labeled toxin is administered intravenously in a range of 5-50 nmoles, preferably about 25 nmoles. The binding of the labeled MII or U002 is then detected by standard techniques well known in the art, such as with the use of a photoscanning device. Although the labeled toxins will bind to SCLC cells, they may also bind to autonomic ganglia. However, the locations of autonomic ganglia are known and can be distinguished from signals resulting from binding of the labeled toxin to SCLC cells.

EXAMPLES

The present invention is described by reference to the following Examples, which are offered by way of illustration and are not intended to limit the invention in any manner. Standard techniques well known in the art or the techniques specifically described below were utilized.

EXAMPLE 1

Biological Activity of U002

The biological activity of conotoxin peptide U002, prepared as described in WO 95/11256 (18), was determined by measuring its effect on spontaneous mepps of frog cutaneous pectoris muscle and by measuring its ability to compete with α-bungarotoxin.

To test whether U002 affected acetylcholine receptors, the toxin's effects on spontaneous miniature end plate potentials (mepps) were measured. Spontaneous miniature end-plate potentials (mepps) were recorded intracellularly from cutaneous pectoris muscle pinned to Sylgard-coated glass cover slips and then placed in a chamber which was secured to the stage of a fluorescence microscope. Toxin was focally applied in a solution containing a tetramethylrhodamine-lysozyme

conjugate (5 μM). The fluorescence of the solution allowed its location to be monitored to be sure that end-plate regions were contacted by the toxin expelled from the puffer pipet. Toxin was washed away from the end plate following withdrawal of the puffer pipet by perfusing the bath. U002 (205 mM) applied from a puffer pipet reduced mepp amplitudes 30%, which recovered following washout of the toxin. This result indicates that acetylcholine receptors were reversibly blocked by the toxin.

Electrophysiological data were acquired with virtual instrument software (LabVIEW National Inst.) on Macintosh computers fitted with A/D converter hardware either from National Instruments (Lab NB) or GW Instruments (Mac-ADIOS adio).

The results with the frog neuromuscular junction, i.e., the blockade of mini-end-plate potentials (mepps) described above, are strongly suggestive (but not conclusive evidence) that this peptide binds to the acetylcholine receptor at the vertebrate neuromuscular junction. In order to confirm this receptor assignment, an investigation was made as to whether α-conotoxin peptide U002 bound to the ligand binding site of the nicotinic acetylcholine receptor by assaying for the ability of this peptide to compete with radiolabeled α-bungarotoxin to this site.

Binding experiments were done using a filtration assay of a post-synaptic membrane fraction isolated from electroplax of Torpedo Californica. The ^{125}I-bungarotoxin (2,000 Ci/mmol) was used at ca. 10^5 cpm per assay; 100% binding under the condition used was 20,000 cpm. Non-specific binding, determined by preincubation with 1 μM unlabeled α-bungarotoxin, was subtracted out. The results of this experiment demonstrated that α-conotoxin peptide U002 at 50 μM blocked 70% of the binding of ^{125}I-α-bungarotoxin to the well-characterized nicotinic acetylcholine receptor found in the Torpedo electric organ.

EXAMPLE 2

Purification of α-Conotoxin MII Peptide

α-Conotoxin MII was isolated from the venom of Conus magus by screening for peptides which bind to neuronal nicotinic receptors of the $\alpha_3\beta_2$ subtype. (MII was alternatively produced as described in WO 95/11256 (18).) Cloned DNA from rat brain encoding neuronal nicotinic receptors was used to make mRNA which was injected into Xenopus oocytes by standard techniques. This mRNA was expressed in the oocytes and the normal nicotinic receptor was made and formed part of the cell surface of the oocytes. Direct physiological effects of acetylcholine can

be made on these cells. MII was found by electrophysiologically screening venom fractions against the cloned nicotinic receptors expressed in Xenopus oocytes. The assay is a standard one. The oocytes are voltage clamped and acetylcholine is added to the media containing the cells. With no toxin present the addition of acetylcholine causes a negative current which can be seen by a current tracing. The presence of a toxin will diminish or abolish this effect. Various snail venoms were tested and venom from Conus magus was found to be especially potent in this assay. This venom was fractionated by HPLC and the different fractions were assayed by this method. Conotoxin MII was isolated as being the active toxin. Conotoxin MII was found to antagonize the effects of acetylcholine. Thus it behaves similarly to all other α-conotoxins in the sense that it is a nicotinic acetylcholine receptor antagonist. However, MII differs from other α-conotoxins in that it potently targets the $\alpha_3\beta_2$ subtype of nicotinic receptor. It also shows potent activity at the α_7 subtype of nicotinic receptor. α-Conotoxin MII blocks the response to acetylcholine in oocytes expressing $\alpha_3\beta_2$ nicotinic acetylcholine receptors with an IC_{50} of 5-10 nM. This peptide is 2-3 orders of magnitude less active in blocking acetylcholine responses of all other tested $\alpha_x\beta_y$ nicotinic acetylcholine receptors.

EXAMPLE 3

Inhibition of SCLC Proliferation by α-Conotoxins

As previously noted, small-cell lung carcinoma (SCLC) cells express cholinergic nicotinic receptors, which are of the neuronal type. Nicotine and cytosine each stimulate the release of 5-hydroxytryptamine (5HT or serotonin) which acts as a potent mitogen in SCLC cells. α-Conotoxin MI blocks the nicotine or cytosine induced release of serotonin and at a concentration of 1 μM it completely antagonized the nicotine and cytosine stimulation of SCLC proliferation. Although α-conotoxin MI binds to these SCLC receptors, α-conotoxin MI is not suitable for therapeutic or diagnostic use since it also binds to neuromuscular receptors and can cause paralysis which could lead to death. α-Conotoxins MII and U002 have a much lower affinity for neuromuscular receptors as compared to the nicotinic neuronal receptors. The activity of α-Conotoxins MII and U002 on SCLC cells is tested as described in Codignola et al. (24). These peptides block the nicotine or cytosine induced release of serotonin and antagonize the nicotine and cytosine stimulation of SCLC proliferation. Thus, MII and U002 are suitable for preventing the

proliferation of tumors such as SCLC and can be used therapeutically to inhibit such proliferation as described below. These α-conotoxins can also be used diagnostically for detecting the presence and/or location of small-cell lung tumors as described below.

EXAMPLE 4

Diagnosis of SCLC Using α-Conotoxins

α-Conotoxins MII or U002 is labeled with ^{131}I or ^{125}I using standard techniques well known in the art. The labeled toxin is administered intravenously in a range of 5-50 nmoles, preferably about 25 nmoles. The label is then detected by standard techniques well known in the art, such as with the use of a photoscanning device. The labeled toxin binds to SCLC cells. In addition, some binding to autonomic ganglia may occur. Signals resulting from binding of the labeled toxin to SCLC cells are easily distinguished from signals resulting from binding of the labeled toxin to autonomic ganglia based on the known locations of the latter.

EXAMPLE 5

Therapeutic Use of α-Conotoxins to Treat SCLC Tumors

α-Conotoxins MII or U002 which bind to SCLC nicotinic receptors and which have lower affinity for neuromuscular receptors are used therapeutically to treat patients with SCLC tumors. A pharmaceutical composition containing MII or U002 is administered, preferably intravenously or intramuscularly, to patients who have been diagnosed with SCLC can have a suitable conotoxin administered. A dose of 200-2000 nanomoles, preferably about 500 nanomoles, is administered. The dosing schedule depends on the *in vivo* stability of the specific conotoxin used. Since the conotoxins are relatively resistant to degradation, they may last on the order of a few days. Therefore a typical dosing schedule for MII or U002 is anywhere from twice per day to once every few days.

It will be appreciated that the methods and compositions of the instant invention can be incorporated in the form of a variety of embodiments, only a few of which are disclosed herein. It will be apparent to the artisan that other embodiments exist and do not depart from the spirit of the invention. Thus, the described embodiments are illustrative and should not be construed as restrictive.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: University of Utah Research Foundation

(ii) TITLE OF INVENTION: Use of Conotoxin Peptides U002 and MII for Treating or Detecting Small-Cell Lung Carcinoma

(iii) NUMBER OF SEQUENCES: 10

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Venable, Baetjer, Howard & Civiletti
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(C) CITY: Washington
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(F) ZIP: 20005

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: MS-WINDOWS
(D) SOFTWARE: Word 6.0

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: WO
(B) FILING DATE: 04-JUN-1996
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/487,174
(B) FILING DATE: 07-JUN-1995

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 202-962-4810
(B) TELEFAX: 202-962-8300

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Conus geographus

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 13
(D) OTHER INFORMATION: /note= "The C-terminus is amidated."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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1 5 10

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Conus geographus

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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1 5 10 15

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Conus geographus

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 13
(D) OTHER INFORMATION: /note= "The C-terminus is amidated."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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1 5 10

(2) INFORMATION FOR SEQ ID NO:4:

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(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Conus magus

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 14
(D) OTHER INFORMATION: /note= "The C-terminus is amidated."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Gly Arg Cys Cys His Pro Ala Cys Gly Lys Asn Tyr Ser Cys
1 5 10

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Conus striatus

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 13
(D) OTHER INFORMATION: /note= "The C-terminus is amidated."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

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1 5 10

(2) INFORMATION FOR SEQ ID NO:6:

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(A) LENGTH: 13 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Conus striatus

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 13
(D) OTHER INFORMATION: /note= "The C-terminus is amidated."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

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1 5 10

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Conus striatus

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 20
(D) OTHER INFORMATION: /note= "Xaa may be des-Xaa or Arg-Thr-Leu."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

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Ser Cys Ser Xaa
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(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: YES

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Conus

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 3..10
(D) OTHER INFORMATION: /note= "Xaa(3) is His or Asn; Xaa(10) is Tyr or Phe."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Cys Cys Xaa Pro Ala Cys Gly Xaa Xaa Xaa Xaa Cys
1 5 10

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Conus imperialis

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 12
(D) OTHER INFORMATION: /note= "The C-terminus is preferably amidated."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

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1 5 10

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Conus magus

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 6
(D) OTHER INFORMATION: /note= "Xaa is Pro or Hydroxy-Pro."

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 16
(D) OTHER INFORMATION: /note= "The C-terminus is preferably amidated."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Gly Cys Cys Ser Asn Xaa Val Cys His Leu Glu His Ser Asn Leu Cys
1 5 10 15

WHAT IS CLAIMED IS:

1. A method for treating a patient having small-cell lung carcinoma which comprises administering a pharmaceutical composition comprising an effective amount of an α-conotoxin selected from the group consisting of MII and U002 in pharmaceutically acceptable carrier.

2. A method for inhibiting small-cell lung carcinoma proliferation which comprises administering a pharmaceutical composition comprising an effective amount of an α-conotoxin selected from the group consisting of MII and U002 in pharmaceutically acceptable carrier.

3. A method for detecting the presence of small-cell lung carcinoma tumors which comprises injecting a subject with a labeled α-conotoxin selected from the group consisting of MII and U002 and detecting the presence of the tumors.

4. A method for detecting the location of small-cell lung carcinoma tumors which comprises injecting a subject with a labeled α-conotoxin selected from the group consisting of MII and U002 and detecting the presence of the tumors.

5. The use of α-conotoxin MII or U002 for the preparation of a pharmaceutical composition for the treatment of a patient having small-cell lung carcinoma.

6. The use of α-conotoxin MII or U002 for the preparation of a pharmaceutical composition for inhibiting small-cell lung carcinoma proliferation.

7. The use of α-conotoxin MII or U002 for the preparation of a diagnostic composition for detecting the presence or location of small-cell lung carcinoma tumors.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/07962

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 38/10
US CL : 514/13, 14
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/2, 13, 14; 435/7.23; 436/64, 813; 530/300, 324, 325, 326

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A,P	CARTIER et al. A new α-conotoxin which targets $\alpha 3\beta 2$ nicotinic acetylcholine receptors. 29 March 1996, J. Biol. Chem. Vol. 271, no. 13. pages 7522-7528, especially Table 1.	1-7
A, P	US 5,514,774 A (B.M. OLIVERA ET AL.) 07 May 1996, column 4, lines 46-48	1-7
A	CODIGNOLA et al. Serotonin release and cell proliferation are under the control of α-bungarotoxin sensitive nicotinic receptors in small cell lung carcinoma cell lines. FEBS Letters. 1994, Vol. 342, No.3, pages 286-90, especially page 289, section 3.4.	1-7

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 01 AUGUST 1996	Date of mailing of the international search report 30 SEP 1996
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer GABRIELE E. BUGAISKY Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/07962

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A, P	CODIGNOLA et al. α-conotoxin imperialis I inhibits nicotine-evoked hormone release and cell proliferation in human neuroendocrine carconoma cells. Neuroscience Letters. 1996, Volume 206, No. 1, pages 53-56, especially page 54, paragraph 1.	1-7

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/07962

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, DIALOG files 155, 5, 350, 351 (Medline, Biosis, Derwent World Patent Index)
search terms: Conotoxin?, cancer, carcinoma, nicotin? (3n) receptor?, neuromuscular junction